Renewable Energy 139 (2019) 1281–1289

Contents lists available at ScienceDirect

Renewable Energy

journal homepage: www.elsevier.com/locate/renene

A strategy for sequential fermentation by *Saccharomyces cerevisiae* and *Pichia stipitis* in bioethanol production from hardwoods

Younho Song [a], Eun Jin Cho [a], Chan Song Park [b], Chi Hoon Oh [b], Bok-Jae Park [c], Hyeun-Jong Bae [a, b, *]

[a] Bio-energy Research Center, Chonnam National University, Gwangju 500-757, Republic of Korea
[b] Department of Bioenergy Science and Technology, Chonnam National University, Gwangju 500-757, Republic of Korea
[c] Division of Business and Commerce, Chonnam National University, Yeosu 550-749, Republic of Korea

ARTICLE INFO

Article history:
Received 21 November 2018
Received in revised form
26 February 2019
Accepted 7 March 2019
Available online 9 March 2019

Keywords:
Hardwood
HPAC pretreatment
SHF
SSF
Bioethanol

ABSTRACT

Climate change due to global warming has led to the expansion of deciduous forests. Increased product yields from hardwood provide an opportunity for the exploitation of biomass resources, such as bioethanol. In this study, the enzymatic hydrolysis of various hardwoods pretreated using the hydrogen peroxide–acetic acid (HPAC) method was evaluated. Glucose and xylose were fermented sequentially to improve the bioethanol production from hardwood. The HPAC pretreatment significantly reduced lignin and improved the hydrolysis efficiency compared with no treatment. The enhanced hydrolysis efficiency contributed to an increase in the bioethanol productivity, which was similar to that of simultaneous saccharification and fermentation, by reducing the processing time of separate hydrolysis and fermentation. The sequential fermentation of glucose and xylose via *Saccharomyces cerevisiae* and *Pichia stipitis*, respectively, improved ethanol production by approximately 12% over conventional fermentation used with glucose alone. The results suggest that sequential fermentation can improve bioethanol production from hardwood.

1. Introduction

The increasing use of fossil fuels is leading to an increase in greenhouse-gas emissions and consequently, to global warming, among other problems [1]. Climate change due to global warming is having significant impacting biodiversity and the sustainability of various ecosystem [2]. The distribution of forests is influenced greatly by climate change. The tropical belt has expanded latitudinally by 11° towards the Northern Hemisphere [3]. Further, according to the Korea Forest Service, coniferous forests in Korea's temperate zone have declined by 10% in the last 4 years, while deciduous forests have expanded due to a rise in the annual average temperature. Consequently, the amount of hardwood waste produced annually due to forest thinning and management has been increasing, and this waste could be utilized as a recycling resources [4].

Softwood is widely used in the construction, furniture manufacturing, and the paper and pulp industries; hardwood is less useful in these applications because of its short fibers and the presence of many knots. Hence, the identification of ways in which to use discarded hardwood is important. Hardwood has a very high potential for use as biomass in the bioethanol industry. The chemical composition of a hardwood cell wall is primarily cellulose (45–50%), hemicellulose (25–35%) and lignin (18–24%). The high cellulose content of hardwood improves the bioethanol productivity by facilitating the production of large amounts of fermentable sugar via enzymatic hydrolysis [5].

Two factors must be considered during the production of bioethanol from hardwood. The first factor is that the enzymatic hydrolysis efficiency of hardwood is low because of its lignin content [6,7]. Chemically and physically, lignin is closely linked to cellulose and hemicellulose; it provides structural support to the cell wall and creates a physical and chemical barrier [8]. These properties of lignin significantly hamper enzymatic hydrolysis by restricting cellulase accessibility to cellulose and the adsorption of cellulase to lignin. To overcome this problem, various pretreatment methods have been developed to effectively remove lignin and improve hydrolysis efficiency [9,10]. Hydrogen peroxide-acetic acid (HPAC)

* Corresponding author. Department of Bioenergy Science and Technology, Chonnam National University, Gwangju 500-757, Republic of Korea.
E-mail address: baehj@chonnam.ac.kr (H.-J. Bae).

https://doi.org/10.1016/j.renene.2019.03.032
0960-1481/

pretreatment was reported to effectively remove lignin from several biomasses including hardwood [11,12]. Hydrogen peroxide and acetic acid react to produce peracetic acid (PAA), PAA degrades lignin to monomeric phenolic compounds to convert the propane side chains to either a hydroxyl or carboxyl group [13]. The lignin content of HPAC-treated softwood, hardwood, and grass was reduced by approximately 98%, 97%, and 85%, respectively, and the hydrolysis efficiency with HPAC-treated biomass was increased by three times than that of their raw materials [12]. Thus, delignification is a major step in the production of bioethanol from hardwoods.

The second factor to consider during the production of bioethanol is the low productivity [14]. Currently, the most commonly used microorganism for the production of bioethanol is *Saccharomyces cerevisiae*, which has a high tolerance to ethanol and sugar [15]. However, *S. cerevisiae* lacks a metabolic mechanism to convert xylose (the second most abundant sugar following the hydrolysis of hemicellulose from hardwood) to ethanol [16]. Although a xylose-fermenting yeast has been studied actively in metabolic engineering, xylose fermentation remains a challenge because xylitol production and low ethanol yields still need to be overcome [17]. Another yeast, *Pichia stipitis*, has been investigated with regard to its use in the fermentation of C6 and C5 sugars including the fermentation of xylose to ethanol. It was found that *P. stipitis* can produce high yields of ethanol from xylose [18]. However, its use as an industrial strain is limited because it has low tolerance to ethanol and sugar [19]. In addition, co-fermentation using *S. cerevisiae* and *P. stipitis* is difficult for fermentation because the oxygen concentrations required for glucose and xylose fermentation are different [20,21]. Therefore, the development of a xylose fermentation technology to improve productivity is another major step to be taken prior to the production of bioethanol from hardwood.

In this study, we focused on developing solutions to the two issues of hydrolysis efficiency and bioethanol productivity to overcome the challenges of bioethanol production from hardwood. A HPAC pretreatment for delignification and the synergistic effect between cellulase and xylanase were tested to enhance the enzymatic hydrolysis efficiency of hardwood. Sequential fermentation using glucose and xylose via *S. cerevisiae* and *P. stipitis* increased bioethanol productivity over that from conventional fermentation using glucose.

2. Materials and methods

2.1. Preparation of raw material

Fringe (*Chionanthus retusus*), zelkova (*Zelkova serrata*), maple (*Acer palmatum*), chestnut (*Castanea crenata*), and false acacia (*Robinia pseudoacacia*) wood were obtained from an arboretum in Chonnam National University, Gwangju in South Korea. Each hardwood was chopped to a particle size of 1–5 cm^3 using a wood chopping machine.

2.2. HPAC pretreatment of hardwoods

Hardwood samples were treated using the slightly modified HPAC pretreatment method by Wi et al. (2015) [11]. The samples of 10% (w/v) hardwood were treated in HPAC solution (hydroperoxide: acetic acid = 1:1, v/v) and then activated at 85 °C for 2 h. HPAC treated hardwoods were separated from the HPAC solution by filtration, and the sample was washed with flowing tap water to remove the remaining HPAC solution. The washed samples were dried using a lyophilizer.

2.3. Carbohydrate composition analysis of hard woods by gas chromatography (GC)

Each raw and HPAC pretreated samples were treated with 0.25 mL of 72% (v/v) sulfuric acid (H_2SO_4) for 45 min at 30 °C and was diluted with distilled water to 4% (v/v) H_2SO_4. The hydrolysis step was performed at 121 °C for 1 h, and a solution containing a known amount of myo-inositol was used as an internal standard. The solution was then neutralized with ammonia water. A sodium borohydride solution (1 mL) and 0.1 mL of glacial (anhydrous) acetic acid (18 M) were added in order to degrade the sodium tetrahydroborate. Then, 0.2 mL of methyl imidazole and 2 mL of anhydrous acetic acid were sequentially added, and 5 mL of deionized water was then added and extracted with 2 mL of dichloromethane. The samples were analyzed via GC (GC-2010; Shimadzu, Otsu, Japan) using a DB-225 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness, J&W; Agilent, Folsom, CA, USA) operated with helium. The operating conditions were as follows: injector temperature of 220 °C, flame ionization detector (FID) at 250 °C, and an oven temperature of 110 °C for 1.5 min with a constant increase of 10 °C/min to 220 °C.

2.4. Enzymatic hydrolysis

2.4.1. Hydrolysis of non- and pretreated hardwood and optimization of cellulase loading

The non- and pretreated hardwoods of 5% (w/v) substrate were treated in 50 mM sodium citrate buffer (pH 5.0), 0 to 50 FPU g^{-1} substrate of cellulase (celluclast 1.5 L; Novozymes, Denmark) and 0.01% sodium azide. They were then completely suspended in a rotary shaker at 45 °C and 200 rpm for 48 h. All experiments were performed in triplicate.

2.4.2. Synergy effect analysis of xylanase with cellulose

To analyze the synergy effect, 5% (w/v) pretreated hardwood chips were hydrolyzed using cellulase (celluclast 1.5 L; Novozymes, Denmark), and xylanase (endo-1, 4-β-xylanase from *Trichoderma longibrachiatum*, Sigma–Aldrich, USA). The substrate was treated with from 0 to 50 μg/mg xylanase, with 30 FPU/g substrate of cellulase at 45 °C and 200 rpm for 48 h.

2.5. Immunogold labeling assay of xylan to fringe wood

Pieces of fringe wood (1 mm × 1 mm × 5 mm) were embedded in London Resin White (London resin Co., UK). Ultrathin sections of 80 nm were placed on uncoated nickel grids (300 mesh). The grids were washed with potassium phosphate buffered saline (PBS, pH 7.4) and rinsed with distilled water for immunogold labeling. The grids were treated with LM10 primary antibody for 2 days at 4 °C, and further labeled with anti-rat antibody conjugated to 15 nm gold particles for 3 h at room temperature. The grids were then stained with 4% uranyl acetate. The sections were visualized using by the transmission electron microscopy (TEM) at an acceleration voltage of 80 kV.

2.6. Fermentation

2.6.1. Separate hydrolysis and fermentation (SHF)

Enzymatic saccharification was conducted in a 500 mL Erlenmeyer flask with a total working volume of 150 mL at 5% (w/v) substrate with 0.1% (w/v) yeast extract, 0.2% (w/v) peptone, and 0.05 M citrate buffer (pH 5.0). Reaction flasks were run in triplicate with an optimized enzyme dose at 190 rpm for 24 h. For the fermentation with *S. cerevisiae* KCTC 7906, 0.5 g of dry yeast was added as an inoculum to 100 mL of hydrolyzates. Fermentation was

carried out at 30 °C for 24 h with agitation at 190 rpm.

2.6.2. Simultaneous saccharification and fermentation (SSF)

Simultaneous saccharification and fermentation were conducted for pretreated materials in a 150 mL total volume containing 5% (w/v) substrate, 0.1% (w/v) yeast extract, 0.2% (w/v) peptone, 0.5 g of dry yeast (*S. cerevisiae* KCTC 7906), and 0.05 M citrate buffer (pH 5.0) at 37 °C for 48 h in a 500 mL Erlenmeyer flask.

2.7. Pervaporative separation of ethanol

A hollow fiber membrane module with an active layer of hydrophobic PDMS, which was of 0.7 μm thickness on the support surface on a macroporous PEI support, was used as prepared by Airrane (Daejon, Korea). Three hundred milliliters of fermentation medium in a conical flask was circulated at 1.8 L/min, and the fermentation medium was kept at an atmospheric pressure. The permeate side was maintained at below 5 mmHg using a vacuum pump in all experiments. Permeate samples were collected at intervals of 30 min for 4 h in a cold trap immersed in liquid nitrogen.

2.8. Xylose fermentation by Pichia stipitis

The fermentation medium was prepared to add the 0.1% (w/v) yeast extract, 0.2% (w/v) peptone, and 0.05 M citrate buffer (pH 5.0) in feed solution after pervaporation. For the xylose fermentation, 0.5 g of dry yeast (*P. stipitis* KCTC 7228) was added as inoculum to 200 mL of the prepared fermentation medium. Fermentation was carried out at 30 °C for 120 h with agitation at 250 rpm and aeration at 0.25 vvm.

2.9. High-performance liquid chromatography (HPLC) analysis

To estimate the product of hydrolysis, fermentation and pervaporation, HPLC (Waters, Milford, MA) was undertaken with Rezex RPM column (4.6 × 300 mm; Phenomenes, USA). The temperatures of the column and the refractive index detector were maintained at 85 and 40 °C, respectively, and deionized water was added to the mobile phase at a flow rate of 0.6 mL/min.

2.10. Cost analysis of bioethanol production

To estimate the cost of bioethanol production, the modified LCOB production method [22] is evaluated according to the following equation:

$$\mathrm{LCOB}(\$/\mathrm{kg\ bioethanol}) = \frac{B + C + E}{P}, \quad (1)$$

where

B is the cost of biomass, C is the cost of chemical, E is the cost of electricity, and P is the one kilogram products. The costs of biomass, chemicals and yeasts used in bioethanol production are shown in Table 1, and energy balance was given in Table 2.

3. Results and discussion

3.1. Chemical composition of five types of hardwood before and after pretreatment

Lignocellulosic biomasses generally has three main components: cellulose, hemicellulose, and lignin. The constituents differ for various grasses, softwoods, and hardwoods. Generally, grass contains less lignin than do other plants, and softwood has a low xylose and high mannose content because its hemicellulose backbone differs from that of hardwood [23]. Due to the differences in these constituents among plants, various pretreatment methods have been developed for bioethanol production [24].

Pretreatment is required for the effective hydrolysis of the lignocellulosic biomass [11,25]. Cellulose consists of hemicellulose and lignin, with lignin serving as a cross-linking agent. Therefore, cellulase accessibility is significantly reduced in cellulose fiber [8–10]. Consequently, delignification is a very important issue in the production of bioethanol, and some pretreatments can effectively remove the lignin [24,26]. Among them, HPAC pretreatment has been reported to effectively reduce the lignin contents of hardwood (oak), softwood (pine) and grass (rice straw) by approximately 97%, 98% and 85%, respectively [11]. In this study, an HPAC pretreatment was applied to five types of hardwoods (fringe, zelkova, maple, chestnut, and false acasia) and the chemical contents of raw- and HPAC pretreated biomasses were analyzed using gas chromatography (Table 3). The HPAC pretreatment method greatly improves downstream enzymatic hydrolysis of lignocellulosic wood biomass. The HPAC pretreatment also can remove lignin without the use of high temperature or strong acid. In addition the acetic acid and hydrogen peroxide degrades rapidly to harmless substances in the environment where it ends up.

After HPAC pretreatment, dry mass weights of the hardwoods were reduced by approximately 43% from the initial weight. The amounts of some structural sugars, such as hemicellulose components, in the cell walls were reduced by at least 60% after HPAC pretreatment. The component reduced most effectively by pretreatment was lignin, with approximately 96.5% reduction achieved due to the dissolution of insoluble lignin and hemicellulose by the combination of H_2O_2 and acetic acid [27]. The HPAC pretreatment involves mixing H2O2 and acetic acid to form reagents, superoxide and hydroxyl radicals, and peracetic acid (PAA) that effectively removes lignin from lignocellulosic biomass. These chemicals as a strong oxidizing agent are able to open the lignin ring, and generated hydroxonium ion from PAA under acidic conditions attacks lignin by ring hydroxylation [13,28]. The glucose (cellulose unit) composition of the hardwoods increased approximately 1.7-fold over those of the raw materials, and the glucose recovery yield was approximately 96.2%. Therefore, HPAC pretreatment changed the color of the samples from brown to a pulp-like white (Fig. 1A). These results suggest that HPAC pretreatment increased the enzymatic hydrolysis efficiency of the five hardwoods without loss of cellulose and with a lignin removal procedure that was not too harsh for other components.

To measure the effect of HPAC pretreatment on hardwood substrates, enzymatic hydrolysis of the five types of HPAC-pretreated hardwood was performed and the results were compared with those obtained with the raw material. The conversion rates of the hardwoods were approximately 2.8 times greater than those of the raw materials (Fig. 1B). These results demonstrate that the lignin content was a primary factor in the inhibition of hydrolysis. Therefore, HPAC pretreatment of hardwoods can improve bioethanol production because of the increased delignification and hydrolysis efficiency.

3.2. Optimization of enzymatic hydrolysis in hardwoods

The use of enzymes is considered to be a cost-related limitation of bioethanol production. Therefore, the conversion yield of enzymatic hydrolysis needs to be optimized and the enzyme dose need to be reduced [29]. Some factors affecting the conversion yield of enzymatic hydrolysis are related to the substrate [30]. The efficiency, yield, and reaction time of hydrolysis are normally improved at low initial substrate concentrations which, however, lead to low sugar concentrations, rendering the production of large

Table 1
The costs of materials used in bioethanol production.

Raw material		Cost, $/kg bioethanol	
		Conventional	Sequential
Biomass		0.07	0.05
H_2O_2		1.20	0.92
Acetic acid		1.20	0.92
Enzyme	Cellulase	0.36	0.28
	Xylnase	0.07	0.04
Chemical of fermentation		0.28	0.24
Yeast	*S. cerevisiae*	0.18	0.12
	P. stipitis		0.12
Total		3.36	2.69

Table 2
The energy balance of the bioethanol production.

Process	Electricity, MJ ($/kg bioethanol)	
	Conventional	Sequential
HPAC pretreatment	36.8 (0.5)	28.1 (0.4)
Enzymatic hydrolysis	83.7 (1.1)	63.8 (0.8)
Glucose fermentation	23.9 (0.3)	18.2 (0.2)
Pervaporation	–	9.8 (0.1)
Xylose fermentation	–	32.3 (0.4)
Total	99.4 (1.8)	152.2 (1.9)

quantities of bioethanol in a downstream process difficult. On the other hand, a high initial substrate concentration can increase the amount produced from a substrate. However, the conversion rate can be reduced due to the high viscosity, type of substrate, or product inhibition by the cellulolytic enzyme system [31,32]. Therefore, the optimum substrate concentration for producing large quantities of sugar and a high conversion rate must to be established.

To optimize the substrate concentration, five HPAC-pretreated hardwoods with substrate concentrations ranging from 1 to 6% were hydrolyzed using 30 FPU g^{-1} glucan of cellulase (Fig. 2A). Conversion rates of >75% were obtained at initial substrate

Table 3
Chemical composition and recovery of lignocellulosic biomass including five types of hardwoods.

Chemical content % (recovery rate)			Pentose		Hexose				Total sugars	Lignin	Reference
Type	Source	Pretreatment	Arabinose	Xylose	Rhamnose	Mannose	Galactose	Glucose			
Grass	Rice	Raw	3.3	24.2	–	0.7	–	41.3	69.5	15.6	[11]
		HPAC	0.4	28	–	0.2	–	47.1	75.7	5.2	
Softwood	Pine	Raw	2.2	7.9	-	13.9	-	45.8	69.8	32.8	
		HPAC	0.4	7.2	-	12.4	-	74	94	0.9	
Hardwood	Oak	Raw	1.1	19.1	–	3.3	–	42.5	66	26.3	
		HPAC	0.3	21.5	–	2	–	63.5	87.3	1.9	
Hardwood	Fringe	Raw	2.2	18.2	0.6	3.8	1.6	42.5	69.9	25.3	This study
		HPAC	0.2 (5.2%)	17.4 (54.5%)	-	2.1 (31.5%)	-	71.8 (96.3%)	91.5 (74.6%)	1.6 (3.6%)	
	Zelkova	Raw	1.9	17.2	0.8	3.3	2.3	43.5	70	26.4	
		HPAC	0.2 (6%)	17.3 (57.3%)	–	2.0 (34.5%)	–	67.8 (88.8%)	87.3 (71.1%)	1.6 (3.5%)	
	Maple	Raw	2.7	17.8	0.7	3.8	2.1	43.7	69.8	25.4	
		HPAC	0.2 (4.2%)	15.7 (50.3%)	-	1.1 (16.5%)	-	70.2 (91.6%)	87.2 (71.2%)	1.7 (3.8%)	
	Chestnut	Raw	2.2	13.2	0.6	3.9	1.9	43.4	65.2	27.3	
		HPAC	0.2 (5.2%)	13.4 (57.9%)	–	1.8 (26.3%)	–	69.1 (90.8%)	84.5 (73.9%)	2.8 (5.8%)	
	False acasia	Raw	2.2	15.7	0.6	3.8	1.8	41.9	67	25.2	
		HPAC	0.2 (5.2%)	16.4 (59.5%)	-	2.2 (33.0%)	-	70.8 (96.3%)	89.6 (76.2%)	1.9 (4.3%)	

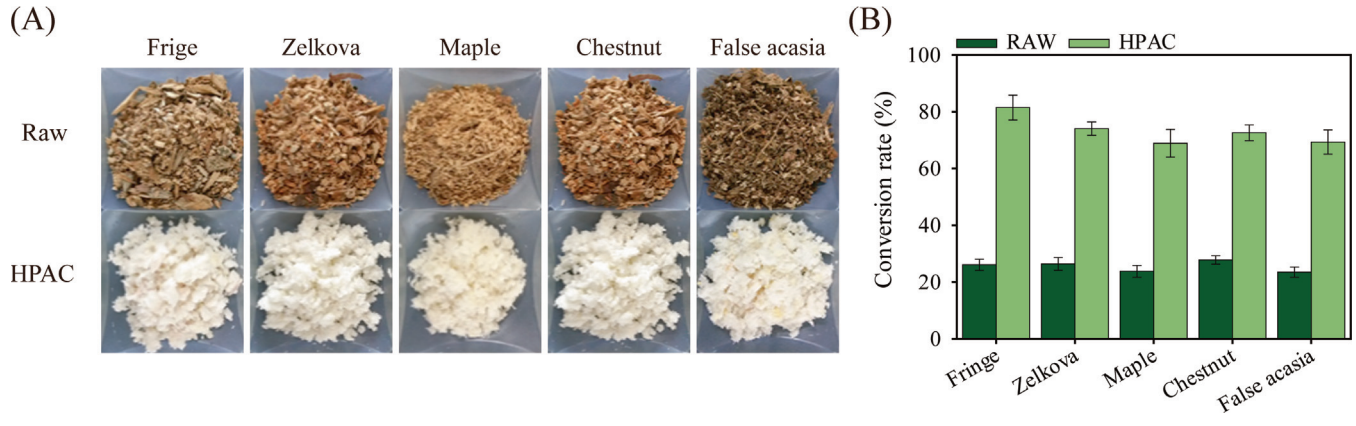

Fig. 1. Comparison of hardwood physical state (A) and saccharification yield (B) before and after HPAC pretreatment.

concentrations of <4%; however, except for the fringe wood, the conversion rates were <75% at initial substrate concentration of >5%. At an initial substrate concentration of 5%, the conversion rate for fringe wood was approximately 83%. The conversion rates of the five hardwoods were <60% at an initial substrate concentration of 6%. These results indicate that an initial substrate concentration of 5% is the optimal condition for obtaining the greatest sugar content, as it yields the maximum conversion rate.

Cost-related factors also impact the effectiveness of enzymatic hydrolysis [33]. Although enzyme prices have been reduced due to advances in production technology, the enzyme loading dose needs to be optimized to improve the price competitiveness of bioethanol [34].

To optimize the enzyme quantity for the hydrolysis of hardwood, five enzyme loadings (10, 20, 30,40, and 50 FPU/g glucan) were applied to the five types of HPAC-pretreated hardwood (Fig. 2B). The conversion rate increased steadily from 0 to 30 FPU/g glucan, but it did not enhance anymore at beyond the cellulase range of 30 FPU/g glucan. These results indicate that increased cellulase loading was not effective beyond a critical point; thus, xylanase was required as an accessory enzyme to improve the conversion rate. The synergistic effect of xylanase with cellulase significantly improved cellulose accessibility by reducing the blocking effect of hemicellulose [35].

To find out the synergistic effect of enzymatic hydrolysis on hardwoods, xylanase were added to 30 FPU cellulase to hydrolyze the five types of HPAC-pretreated hardwoods. Xylanase eliminated the blocking effect by degrading the hemicellulose that serves as a crosslinking agent connecting cellulose to lignin [36]. Treatment with xylanase alone yielded conversion rates of <15% for HPAC-pretreated hardwoods. The synergistic effects of xylanase with cellulase increased the conversion rates by approximately 15% over that from using cellulase alone (Fig. 2C). To confirm the reason that xylanase is required despite the reduction of xylan content by approximately 48% after HPAC pretreatment, the distribution pattern of xylan before and after HPAC pretreatment of fringe wood was analyzed by immunogold labeling using two different monoclonal antibodies, LM10 and LM11, which recognize different epitopes on the xylan chain. LM10 preferably binds to un- or lower-substituted xylan, whereas LM11 binds to both low- and high-substituted xylan. The xylan was mostly distributed in the S1 layer in raw material, but particle density was significantly reduced by approximately 75% after HPAC pretreatment. Rather, particle density in the S2 layer in HPAC pretreatment of fringe wood was relatively increased approximately 2.9-times that of the raw material (Fig. 3A, B, and D). The xylan exposed to HPAC pretreatment showed a high ratio of un- or lower-substituted xylan with a particle density difference of less than 12% between LM10 and LM11 (Fig. 3B, C and E). These results indicated that xylanase alone can effectively remove xylan, which is completely entangled with cellulose, without adding accessory enzymes for substituent removal, and xylanase supplementation is required for the effective enzymatic hydrolysis of HPAC-pretreated hardwood, because it provides for the maintenance of hemicellulose during the HPAC pretreatment.

3.3. Comparison of SHF and SSF processes from HPAC-treated fringe wood

Hydrolysis and fermentation are the main steps taken after pretreatment process to produce bioethanol. Separate hydrolysis and fermentation (SHF) is a universal method that consists of enzymatic hydrolysis followed by yeast fermentation, which provides the optimal condition for bioethanol production. Simultaneous saccharification and fermentation (SSF) is a streamlined method for saccharification and fermentation in a single reactor [37]. Each process has advantages and disadvantages for bioethanol production, such as those related to productivity and the

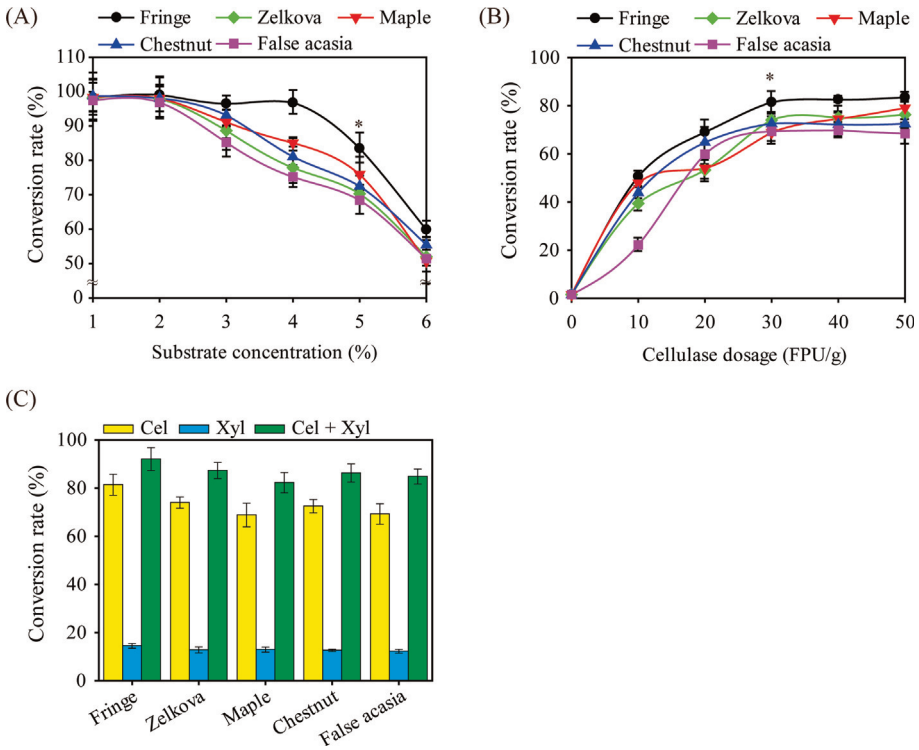

Fig. 2. Optimization of enzymatic hydrolysis on substrate-related and enzyme-related factor. Saccharification profile of HPAC pretreated hardwood hydrolysis with increasing (A) substrate concentration and (B) cellulase loading dose. (C) Synergistic effect analysis of cellulase and xylanase on HPAC pretreated hardwood. Cel; cellulase, Xyl; xylanase.

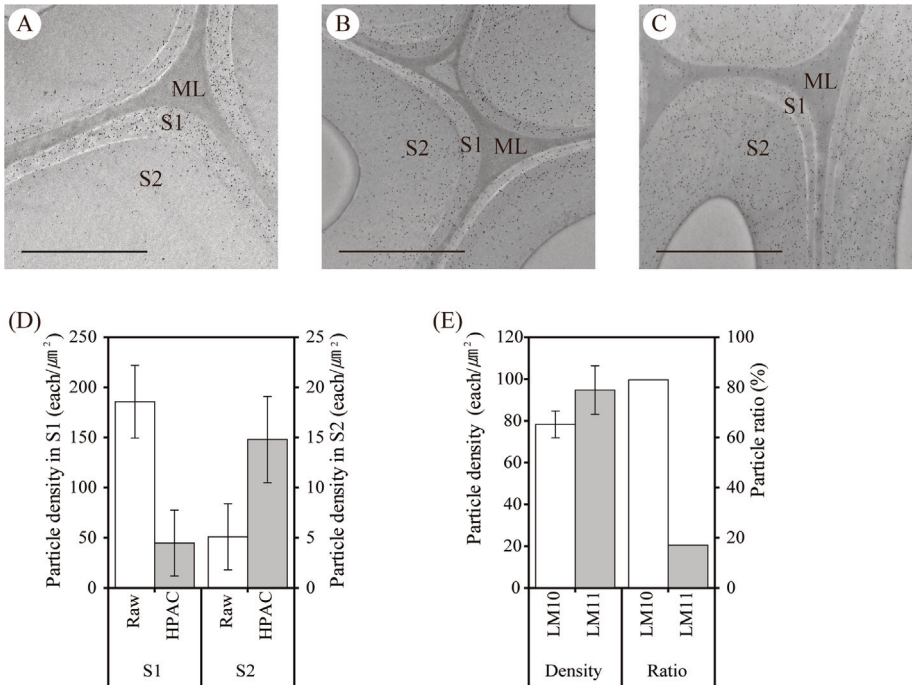

Fig. 3. Immunogold localization of xylan in raw (A) and HPAC pretreated fringe wood (B and C) using LM10 (A and B) and LM11 (C). Quantitative analysis of particle density in secondary cell wall (D) and ratio analysis of LM10 and LM11 with and without substituent (E). Scale bar represents 0.2 μm.

processing time required. To produce bioethanol from fringe wood more effectively, we compared and analyzed the SSF and SHF processes.

The SHF was conducted under the optimal enzymatic hydrolysis (saccharification) condition, 45 °C and pH 5.0, for 24 h, followed by fermentation by *S. cerevisiae* at 30 °C for 24 h (Fig. 4A). After saccharification from the 5% HPAC-pretreated fringe wood, approximately 34.3 mg/mL and 7.4 mg/mL of glucose and xylose were produced with conversion rates of 95.5% and 84.1%, respectively. The released glucose was converted to 14.8 mg/mL ethanol, i.e., 80.7% of the theoretical ethanol yield (per unit weight of biomass), within a 24-h period. SSF was conducted at 37 °C for 48 h (Fig. 4B). The xylose and ethanol concentrations reached approximately 4.1 mg/mL and 15.2 mg/mL, which represented conversion yields of approximately 46.6% and 83.1%, respectively. The ethanol productivity values for SHF and SSF were calculated to be approximately 0.31 g/L/h and 0.32 g/L/h, respectively.

Various fermentation studies have shown that SSF produces ethanol more efficiently, with greater ethanol productivity and yield, than does SHF, although the optimal hydrolysis and fermentation condition have not been identified [38]. These results are considered to result from long saccharification time and feedback inhibition provided by the product [39]. However, productivity was improved, with a short hydrolysis time of 24 h, when HPAC-pretreated fringe wood was used for SHF. Productivity differed from that of SSF by < 5%. In addition, xylose production was approximately 1.8-fold greater in SHF than in SSF. *Saccharomyces cerevisiae* cannot use xylose, an unfermented sugar, to produce bioethanol [17,40]. Metabolically engineered *S. cerevisiae*, has recently been used to improve bioethanol production from xylose,

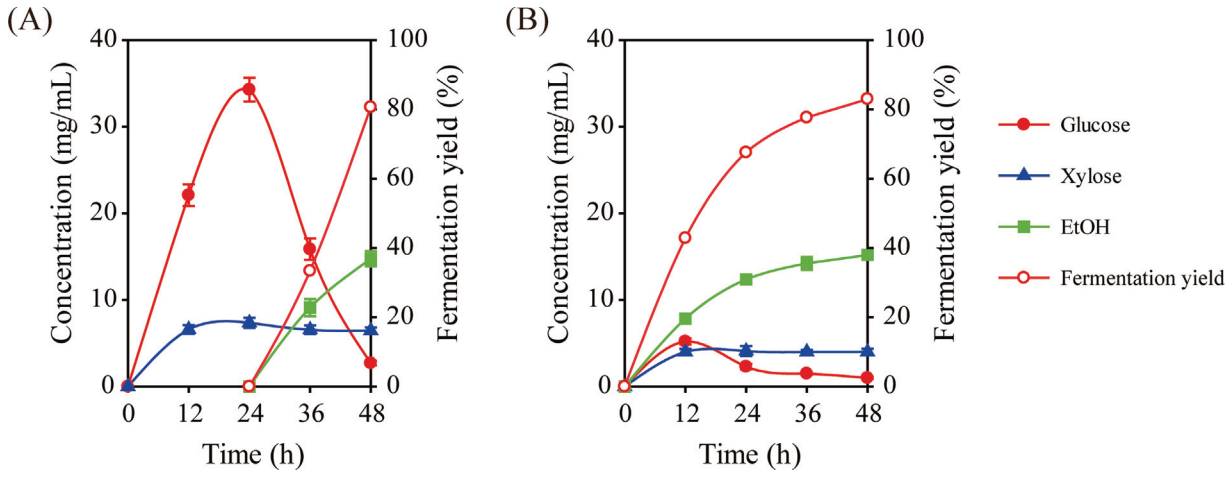

Fig. 4. Time courses of (A) SHF and (B) SSF via *S. cerevisiae* for ethanol production from HPAC pretreated fringe wood.

but challenges remain about the xylose fermentation of xylitol and low yields of ethanol production [17]. In this study, we attempted to produce a bioethanol from xylose using *P. stipites*, a strain optimized for sugar fermentation.

3.4. Separation of bioethanol and xylose from the fermentation medium by pervaporation and xylose fermentation by P. stipitis

Above mentioned, use of *P. stipitis* as an industrial strain in bioethanol production is limited because it has low tolerance to ethanol and sugar. [19]. Therefore, xylose and ethanol must be separated for xylose fermentation to avoid this problem. Xylose and ethanol were separated from the fermentation medium using pervaporation. A composite polydimethylsiloxane/polyetherimide (PDMS/PEI) hollow-fiber membrane, reported to yield good total flux values and to have high-quality separation factors, was used for the pervaporation experiments [41].

Fig. 5A shows the process according to the time of ethanol separation from the fermentation medium using the membrane. The initial concentrations of xylose and ethanol in a 300 mL volume of fermentation medium, used as feed solution were 6.5 and 14.8 mg/mL, respectively. During circulation at a flow rate of 1.8 L/min for 240 min, approximately 37.9 mg/mL of ethanol was harvested from a 100 mL product solution. The recovery yield of ethanol was approximately 85.3%. The xylose in the feed solution was concentrated approximately 1.3 times due to the evaporation of the solution. However, the recovery of xylose was approximately 86.7%.

After pervaporation, xylose was fermented using *P. stipitis* to confirm that the remaining xylose in the feed solution was converted to ethanol. The xylose was converted to 3.7 mg/mL of ethanol after 120 h at 76.3% of the theoretical ethanol yield (Fig. 5B). In addition, ethanol production by the sequential fermentation of glucose and xylose was increased by approximately 12% over that of conventional fermentation.

These results indicate that the pervaporation can avoid the tolerance problems of *P. stipites,* encountered with high concentrations of ethanol and sugars, and that xylose fermentation of hardwood can improve bioethanol productivity.

3.5. Overall mass balance and cost analysis

Based on composition analysis, an overall mass balance diagram was produced for each step in the process, including the HPAC pretreatment, enzymatic hydrolysis, SHF by *S. cerevisiae*, pervaporation, and xylose fermentation by *P. stipites* (Fig. 6). One kilogram of fringe wood containing 425 g glucose and 182 g xylose was reduced to 550 g dry mass after HPAC pretreatment, and 394 g and 93.9 g of glucose and xylose were harvested, with recovery yield of 92.9% and 51.6%, respectively. During enzymatic hydrolysis using cellulase and xylanase, approximately 377.1 g glucose and 79.0 g xylose, respectively, were produced in the hydrolyzate. The fermentation of hydrolyzates using *S. cerevisiae* resulted in their effective conversion to 155.2 g of ethanol, with a fermentation yield of approximately 80.7% and 69.3 g xylose remaining. The products were separated by pervaporation, and 132.4 g ethanol was collected as a phase liquor product, and 60.1 g xylose and 20.0 g of ethanol remained in the feed phase solution. During fermentation using *P. stipits*, xylose was converted to 23.4 g of ethanol, with a fermentation yield of approximately 76.3%.

Based on the above mass balance, the bioethanol production costs were estimated between conventional and sequential fermentation. The estimation of the production cost is calculated mainly based on the variable cost. The levelised cost of bioethanol (LCOB) of conventional and sequential fermentation from hardwood wastes was estimated approximately 5.16 $/kg and 4.59 $/kg, respectively. These data indicate that sequential fermentation can reduce the production costs compared to conventional method by using less biomass to produce the same amount of products, although energy use and time have increased.

4. Conclusion

The production of bioethanol from hardwoods, including fringe wood, is difficult due to the low level of enzymatic hydrolysis caused by the high lignin contents, despite the high sugar content. HPAC pretreatment effectively removed the lignin from the fringe wood, and increased the hydrolysis efficiency by 3.1-fold over that of the raw material. When xylanase was used together with cellulase, the conversion rate of hardwood increased from 82% to about 98% due to the synergistic effect. SHF using *S. cerevisiae* lead to efficient conversion of released glucose (~84.5%), and ethanol and xylose were separated by pervaporation. Pentose fermentation using *P. stipites* converted approximately 76.3% of the separated xylose. The production of bioethanol was increased by approximately 12% over that from conventional fermentation. The technology to produce bioethanol from wood based by-products is far from being optimized. In specifically, efficiently metabolize process development C6 and C5 sugars is still needed in fermentation strategies and being overcome. For these reason, this strategy of

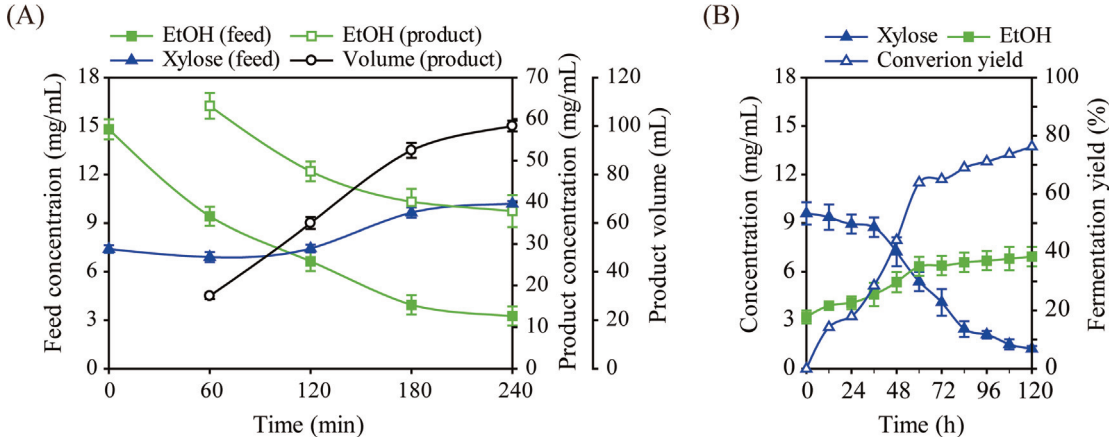

Fig. 5. Time course of (A) pervaporative separation of ethanol and xylose from fermentable media and (B) ethanol production from remained xylose in the feed solution via *P. stipitis*.

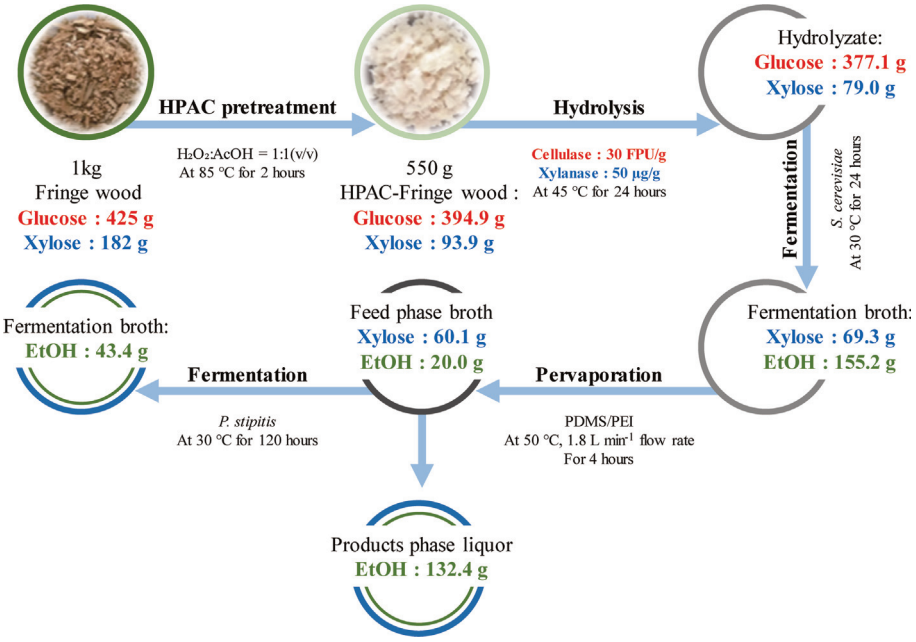

Fig. 6. Overall mass balance for bioethanol production from fringe wood.

sequential fermentation enhanced the potential of hardwood biomass for bioethanol productivity, despite the previously reported difficulties in producing bioethanol from hardwood.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2017R1A6A3A01006154) and Basic Science Research Program (NRF-2018R1a2a2a05018238) through the National Research Foundation (NRF) of South Korea funded by the Ministry of Education.

References

[1] N. Heidari, J.M. Pearce, A review of greenhouse gas emission liabilities as the value of renewable energy for mitigating lawsuits for climate change related damages, Renew. Sustain. Energy Rev. 55 (2016) 899–908.
[2] D. Thom, W. Rammer, T. Dirnböck, J. Müller, J. Kobler, K. Katzensteiner, N. Helm, R. Seidl, The impacts of climate change and disturbance on spatio-temporal trajectories of biodiversity in a temperate forest landscape, J. Appl. Ecol. 54 (2017) 28–38.
[3] A. Rajaud, N. de Noblet-Ducoudré, Tropical semi-arid regions expanding over temperate latitudes under climate change, Clim. Change 144 (2017) 703–719.
[4] M.H. Kim, H.B. Song, Analysis of the global warming potential for wood waste recycling systems, J. Clean. Prod. 69 (2014) 199–207.
[5] S.R. Pereira, D.J. Portugal-Nunes, D.V. Evtuguin, L.S. Serafim, A.M.R.B. Xavier, Advances in ethanol production from hardwood spent sulphite liquors, Process Biochem. 48 (2013) 272–282.
[6] J.K. Ko, Y. Kim, E. Ximenes, M.R. Ladisch, Effect of liquid hot water pretreatment severity on properties of hardwood lignin and enzymatic hydrolysis of cellulose, Biotechnol. Bioeng. 112 (2015) 252–262.
[7] M. Raud, M. Tutt, T. Kikas, Dependence of the hydrolysis efficiency on the lignin content in lignocellulosic material, Int. J. Hydrog. Energy 41 (2016) 16338–16343.
[8] C. Qin, K. Clarke, K. Li, Interactive forces between lignin and cellulase as determined by atomic force microscopy, Biotechnol, Biofuels 7 (2014) 65.
[9] G. Siqueira, V. Arantes, J.N. Saddler, A. Ferraz, A.M.F. Milagres, Limitation of cellulose accessibility and unproductive binding of cellulases by pretreated sugarcane bagasse lignin, Biotechnol. Biofuels 10 (2017) 176.
[10] J.K. Saini, A.K. Patel, M. Adsul, R.R. Singhania, Cellulase adsorption on lignin: a roadblock for economic hydrolysis of biomass, Renew. Energy 98 (2016) 29–42.
[11] S.G. Wi, E.J. Cho, D.S. Lee, S.J. Lee, Y.J. Lee, H.J. Bae, Lignocellulose conversion for biofuel: a new pretreatment greatly improves downstream biocatalytic hydrolysis of various lignocellulosic materials, Biotechnol. Biofuels 8 (2015) 228.
[12] Y. Song, S.G. Wi, H.M. Kim, H.J. Bae, Cellulosic bioethanol production from Jerusalem artichoke (*Helianthus tuberosus* L.) using hydrogen peroxide-acetic acid (HPAC) pretreatment, Bioresour. Technol. 214 (2016) 30–36.
[13] R. Ma, M. Guo, K.T. Lin, V.R. Hebert, J. Zhang, M.P. Wolcott, M. Quintero, K.K. Ramasamy, X. Chen, X. Zhang, Peracetic acid depolymerization of biorefinery lignin for production of selective monomeric phenolic compounds, Green Chem. 22 (2016) 10884–10891.
[14] J. Baeyens, Q. Kang, L. Appels, R. Dewil, Y. Lv, T. Tan, Challenges and opportunities in improving the production of bio-ethanol, Prog. Energy Combust. Sci. 47 (2015) 60–88.
[15] S.H. Mohd Azhar, R. Abdulla, S.A. Jambo, H. Marbawi, J.A. Gansau, A.A. Mohd Faik, K.F. Rodrigues, Yeasts in sustainable bioethanol production: a review, Biochem. Biophys. Rep. 10 (2017) 52–61.
[16] D.B. Jordan, M.J. Bowman, J.D. Braker, B.S. Dien, R.E. Hector, C.C. Lee, J.A. Mertens, K. Wagschal, Plant cell walls to ethanol, Biochem. J. 442 (2012) 241–252.
[17] D.N. Moysés, V.C. Reis, J.R. de Almeida, L.M. de Moraes, F.A. Torres, Xylose fermentation by *Saccharomyces cerevisiae*: challenges and prospects, Int. J. Mol. Sci. 17 (2016) 207.
[18] H.G. Yücel, Z. Aksu, Ethanol fermentation characteristics of *Pichia stipitis* yeast from sugar beet pulp hydrolysate: use of new detoxification methods, Fuel 158 (2015) 793–799.
[19] J. Shi, M. Zhang, L. Zhang, P. Wang, L. Jiang, H. Deng, Xylose-fermenting Pichia stipitis by genome shuffling for improved ethanol production, Microb. Biotechnol. 7 (2014) 90–99.
[20] P. Morales, V. Rojas, M. Quirós, R. Gonzalez, The impact of oxygen on the final alcohol content of wine fermented by a mixed starter culture, Appl. Microbiol. Biotechnol. 99 (2015) 3993–4003.
[21] T.H. Lin, G.L. Guo, W.S. Hwang, S.L. Huang, The addition of hydrolyzed rice straw in xylose fermentation by *Pichia stipitis* to increase bioethanol production at the pilot-scale, Biomass Bioenergy 91 (2016) 204–209.
[22] I. Hannula, Co-production of synthetic fuels and district heat from biomass residues, carbon dioxide and electricity: performance and cost analysis, Biomass Bioenergy 74 (2015) 26–46.
[23] Z. Anwar, M. Gulfraz, M. Irshad, Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy: a brief review, J. Radiat. Res. Appl. Sci. 7 (2014) 163–173.
[24] S.H. Mood, A.H. Golfeshan, M. Tabatabaei, G.S. Jouzani, G.H. Najafi, M. Gholami, M. Ardjmand, Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment, Renew. Sustain. Energy Rev. 27 (2013) 77–93.
[25] S. Sun, S. Sun, X. Cao, R. Sun, The role of pretreatment in improving the enzymatic hydrolysis of lignocellulosic materials, Bioresour. Technol. 199 (2016) 49–58.
[26] R. Singh, A. Shukla, S. Tiwari, M. Srivastava, A review on delignification of lignocellulosic biomass for enhancement of ethanol production potential, Renew. Sustain. Energy Rev. 32 (2014) 713–728.
[27] D. Watkins, M. Nuruddin, M. Hosur, A. Tcherbi-Narteh, S. Jeelani, Extraction and characterization of lignin from different biomass resources, J. Mater. Res. Technol. 4 (2015) 26–32.

[28] O. Sánchez, R. Sierra, C.J. Alméciga-Díaz, in: M. Manzanera (Ed.), Delignification Process of Agro-Industrial Wastes an Alternative to Obtain Fermentable Carbohydrates for Producing Fuel, Alternative Fuel, InTech, 2011, pp. 111–154.
[29] D. Klein-Marcuschamer, P. Oleskowicz-Popiel, B.A. Simmons, H.W. Blanch, The challenge of enzyme cost in the production of lignocellulosic biofuels, Biotechnol. Bioeng. 109 (2012) 1083–1087.
[30] S.Y. Leu, J.Y. Zhu, Substrate-related factors affecting enzymatic saccharification of lignocelluloses: our recent understanding, BioEnergy Res. 6 (2013) 405–415.
[31] A.A. Modenbach, S.E. Nokes, Enzymatic hydrolysis of biomass at high-solids loadings – a review, Biomass Bioenergy 56 (2013) 526–544.
[32] L.J. Jönsson, C. Martín, Pretreatment of lignocellulose: formation of inhibitory by-products and strategies for minimizing their effects, Bioresour. Technol. 199 (2016) 103–112.
[33] J.B. Kristensen, C. Felby, H. Jørgensen, Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose, Biotechnol. Biofuels 2 (2009) 11.
[34] A.C. Neto, M.J.O.C. Guimarães, E. Freire, Business models for commercial scale second-generation bioethanol production, J. Clean. Prod. 184 (2018) 168–178.
[35] J. Hu, V. Arantes, J.N. Saddler, The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect? Biotechnol. Biofuels 4 (2011) 36.
[36] Y. Song, Y.G. Lee, I.S. Choi, K.H. Lee, E.J. Cho, H.J. Bae, Heterologous expression of endo-1,4-β-xylanase A from *Schizophyllum commune* in *Pichia pastoris* and functional characterization of the recombinant enzyme, Enzym. Microb. Technol. 52 (2013) 170–176.
[37] M. Ask, K. Olofsson, T.D. Felice, L. Ruohonen, M. Penttilä, G. Lidén, L. Olsson, Challenges in enzymatic hydrolysis and fermentation of pretreated Arundo donax revealed by a comparison between SHF and SSF, Process Biochem. 47 (2012) 1452–1459.
[38] E. Domínguez, A. Romaní, L. Domingues, G. Garrote, Evaluation of strategies for second generation bioethanol production from fast growing biomass Paulownia within a biorefinery scheme, Appl. Energy 187 (2017) 777–789.
[39] N. Sarkar, S.K. Ghosh, S. Bannerjee, K. Aikat, Bioethanol production from agricultural wastes: an overview, Renew. Energy 37 (2012) 19–27.
[40] S. Kwak, Y.S. Jin, Production of fuels and chemicals from xylose by engineered Saccharomyces cerevisiae: a review and perspective, Microb. Cell Factories 16 (2017) 82.
[41] H.J. Lee, E.J. Cho, Y.G. Kim, I.S. Choi, H.J. Bae, Pervaporative separation of bioethanol using a polydimethylsiloxane/polyetherimide composite hollow-fiber membrane, Bioresour. Technol. 109 (2012) 110–115.